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14. ABSTRACT:

Sex hormone-binding globulin (SHBG) is a plasma protein that binds androgens. It also is a transducer of androgen signaling at the plasma membrane of prostate cancer cells. We have found that the human prostate cancer cell line, LNCaP, in addition to having a receptor for SHBG (RSHBG), produces its own SHBG. We hypothesize that local regulation of SHBG production and/or secretion results in important autocrine and paracrine effects that influence gene expression and growth in prostate cancer cells.

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Table of Contents

Progress report: "Unique Approaches to Androgen Effects on Prostate Cancer"

INTRODUCTION

Background: Androgens are central both to inception and progression of prostate cancer. Individuals deprived of androgens early in life, such as in castration in youth or in the syndrome of 5α reductase deficiency, do not get prostate cancer. Further, after prostate cancer is established, androgen deprivation causes temporary remission/improvement in the majority of patients. These two clinical facts are not fully explained by our current understanding of how androgens exert their effects.

We have shown the plasma protein sex hormone-binding globulin (SHBG) not only binds testosterone (T) and other androgens in plasma, but is part of a prostatic androgen signal transduction system that starts with a receptor (R_{SHBG}) for SHBG on prostate cell membranes (1). The SHBG-R_{SHBG} complex is activated by an appropriate steroid hormone, such as estradiol (E_2) or 5α-androstan-3α, 17β-diol (3αdiol) (forming the new complex, 3αdiol-SHBG-R_{SHBG}) that triggers a second messenger system, via cAMP. Furthermore, in whole non-cancerous explants of human prostate, the system can cause increases in the secretion of prostate specific antigen (PSA) (2), an event previously thought to be related only to activation of the AR by T or dihydrotestosterone. Our general understanding of the system was based on the assumption that SHBG (which, like most plasma proteins, is produced in the liver) arrived at the prostate only by way of the plasma. We now have shown that prostate cancer cells stain with anti-SHBG antibodies and, more importantly, that a number of prostate cancer cell lines (LNCaP, PC-3, and DU-145) contain both SHBG mRNA and SHBG protein (3). The expression of SHBG by prostate cancer cells raises the important question of how local regulation of SHBG synthesis might function either to act on the sequestration of steroid hormones within the prostate or to alter androgen induced signal transduction in an autocrine or paracrine fashion.

Objective Hypothesis: We propose that the expression of SHBG by prostate cancer cells is biologically regulated and that this SHBG functions to alter the effects of androgens and estrogens within the prostate cancer cell.

Specific Aims: (Aim 1) Generate prostate cancer cell lines that stably express SHBG in a regulatable fashion. Examine (Aim 2) autocrine and (Aim 3) paracrine effects of prostate SHBG synthesis on steroid signaling. (Aim 4) Examine the effect of SHBG synthesis within the prostate on the growth of prostate cancer cell lines both in the presence and absence of androgen.

Experimental Design: We will undertake genetically and pharmacologically based studies to address our hypothesis. Prostate cancer cells from Aim 1 will be exposed to steroids that selectively stimulate either the androgen receptor or the SHBG-receptor based pathways. Further, although AR and SHBG both are high affinity binders of testosterone and DHT, there are other ligands that are specific to each. The same situation exists for inhibitors of each of the two systems. We will independently stimulate and/or inhibit

each of these two signaling systems with such ligands. This pharmacologic approach will allow us to dissect the influence of androgens on these two pathways and further ascertain how each contributes to the growth of prostate cancer cells.

BODY

Our major work over the past year focused on two major aspects of local SHBG expression in prostate cells. To better understand the biologic function of locally expressed SHBG, we devoted much effort to a detailed characterization of SHBG gene expression. In addition, we performed microarray analyses to examine the effects of SHBG gene expression on overall gene transcription in LNCaP cells, since this technology is more powerful and informative than the reporter assays that we had originally planned.

SHBG gene expression

Over this past year, we pursued our initial findings that the SHBG gene has a much more complex transcription pattern than was previously reported. Previously, two major human SHBG genes transcripts had been reported (4)(Figure 1A), one encoding the secreted form of SHBG and the second encoding an alternative transcript lacking exon 7 that originated from a separate promoter and was most abundant in testis. We now have a more comprehensive overall picture of how the human SHBG gene is expressed (Figure 1B).

The SHBG gene has at least three active promoters. The downstream promoter regulates expression of at least eight different SHBG transcripts, including the secreted form of SHBG found in plasma. The intermediate promoter, located 2 kb upstream, regulates expression of at least four main SHBG transcripts, including the major species found in testis. The third, and novel promoter discovered as a part of these studies, is located a further15 kb upstream, and we have found it to regulate expression of at least four main different SHBG transcripts. The two upstream promoters map to promoters for genes that are adjacent to the SHBG gene, and are transcribed in the opposite direction. Apparently, structural and/or transcription factors allow the SHBG gene to utilize these promoters, making them bi-directional. Sequence analysis of the transcripts that originate from the novel, third upstream promoter, reveals a first exon that, after encountering the first ATG codon, contains a nine amino acid open reading frame, followed by a termination codon. It is curious that the termination codon is followed by a long open reading frame that merges within exon 2 with the open reading frame of the secreted form of SHBG. We are very interested in understanding whether translation stops at this termination codon, or if the translation machinery can hiccup past.

Real-time PCR experiments were performed to quantitate SHBG transcriptional activity in the LNCaP prostate cancer cell line and normal prostate tissue. Expression in the prostate was compared to the liver (HepG2 cancer cell line and normal liver tissue), testis (testis tissue), and breast (MCF-7, 184, and MB231 cancer cell lines and normal breast

tissue). Total SHBG gene transcription was measured by quantitative PCR using primers specific for exons 2 and 3, both of which are conserved amongst all known SHBG gene transcripts (Figure 2A). The highest SHBG expression levels were in testis, the HepG2 cell line, and normal liver tissue. LNCaP cells had approximately a seven-fold higher expression level than normal prostate tissue. Normal breast tissue had just over a two-fold higher expression level than normal prostate tissue, and SHBG expression was also present at similar levels in the MCF-7, and MB231 breast cell lines and slightly higher in the 184 cell line. These results are to be expected from the physiology of SHBG. The liver needs to make large amounts of SHBG to populate the plasma; the testes needs to secrete relatively large amounts of SHBG into the tubules; the prostate and breast, on the other hand, need make only small amounts for local, e.g. paracrine or autocrine use.

SHBG gene expression arising from each of the three individual SHBG gene promoters was quantitated by real-time PCR. Three primer sets were designed, each with an upstream primer specific for either exon 1L, 1T, or 1N, and a downstream primer specific for exon 2. Normal prostate mostly expresses 1L transcripts, though in much lower (140-fold) amounts when compared to the liver. This result is consistent with our hypothesis that locally produced SHBG has an autocrine or paracrine effect on R_{SHBG} signaling within the prostate. Expression of 1T and 1N transcripts are 42% and 81% lower, respectively than expression of 1L transcripts in normal prostate. Interestingly, of all the cell lines and tissues that we have examined, LNCaP cells express the greatest amounts of 1N transcripts. 1T transcripts are 22-fold more abundant in LNCaP cells than in normal prostate, and 1L transcripts are nearly 3-fold more abundant. Whether the increase in overall and specific SHBG transcription levels in LNCaP cells compared to normal prostate tissue is due to cell type, malignancy, or other factors is unclear.

Promoter utilization by the SHBG gene can show tissue specificity (Fig 2B). Although HepG2 cells and normal liver tissue have abundant expression of 1L transcripts, the greatest expression of 1T transcripts was in normal testis tissue. As mentioned before, LNCaP cells expressed the highest level of 1N transcripts. Interestingly, 1N transcripts are exceedingly scarce in the liver. It appears that tumor cell lines express more 1N transcripts than the normal tissue from which they were derived. Whether 1N transcripts contribute to the malignant phenotype or whether their increased expression results from general genomic instability and deregulation of gene expression in cancer cell lines remains to be determined.

Because our work unexpectedly revealed that alternative splicing of exon 6 occurs in SHBG transcripts, we performed a detailed analysis of SHBG transcript structures by RT-PCR. First, we used a primer set that consisted of an upstream primer specific for exon 2 and a downstream primer specific for exon 8. From this we obtained an overall picture of SHBG gene transcription, regardless of the promoter from which the transcript originated. LNCaP cells gave eight independent RT-PCR bands (Figure 3A), and we were able to reamplify and successfully sequence four of the eight bands. The largest band had contiguous sequences between exons 2 and 8, while the three others corresponded to transcripts lacking exon 7, exons 6 and 7, and exons 4, 6, and 7. Based

on their sizes, we speculate that two of the remaining RT-PCR bands lack exon 6 and exon 4 alone, but this needs to be confirmed. The overall MCF-7 expression pattern appeared similar to that of LNCaP, while HepG2 and testis tissue had different relative band intensities, probably due to tissue specific factors. It appears, however, that the same eight bands present in LNCaP are also present in the other samples tested.

Next, we performed a series of RT-PCR experiments using upstream primers specific for exons 1L, 1T, or 1N, and a downstream primer specific for exon 8. This strategy was designed to enable us to identify SHBG splice variants arising from each individual promoter (summarized in Figure 1B). Our initial experiments using the 1L primer set (Figure 3B) did not generate RT-PCR transcripts for LNCaP cells, and we are currently in the process of repeating these experiments, since the real time quantitative PCR experiments did show that the downstream promoter is active in LNCaP cells. HepG2 and liver gave complex RT-PCR patterns, each producing at least eight distinguishable bands. We have successfully reamplified and sequenced four of the eight HepG2 RT-PCR bands. We found that the largest consisted of contiguous exon 1L-8 sequences. The next two bands, in order of size, have not been reamplified. However based on their sizes, we predict that they reflect SHBG transcripts that lack exon 6 and exon 4, respectively. Sequence analysis of the fourth largest band reveals it to lack exon 7. The band immediately below the exon 7-lacking band has not yet been successfully sequenced. Next in size is a doublet, of which one band lacks exons 6 and 7; the other is not yet sequenced. The smallest RT-PCR band lacks exons 4, 6, and 7. All eight bands appear in normal liver, at different expression levels. The contiguous exon 1L-8 band, which encodes the secreted plasma form of SHBG, appears to predominate. Testis tissue gave a weak pattern; we detected faint bands corresponding to the contiguous exon 1L-8 transcript, the hypothetical exon 6-lacking transcript, and the exon 7-lacking transcript.

In the 1T RT-PCR assay (Figure 3C), LNCaP cells gave rise to four major bands; the largest contains contiguous 1T-8 sequences. The other three bands lacked exons 7, 6 and 7, and 4, 6, and 7, respectively. Testis also produced the same four major bands, and, as expected, the exon 7-lacking band was most intense. Minor species also appear to be present in the testis profile, but these await further characterization. The RT-PCR band lacking exons 4, 6, and 7 was most prominent in the HepG2 sample.

In the 1N RT-PCR assay (Figure 3D), LNCaP cells also gave rise to four major bands. These were similar in structure to the 1T bands. The largest had contiguous exon 1N-8 sequences, while the smaller bands lacked exon 7, exons 6 and 7, and exons 4, 6, and 7, respectively. Minor LNCaP RT-PCR bands are also present, and these await further analysis. HepG2 cells did not give a reproducible RT-PCR pattern, which we speculate is due to the very low expression levels of 1N transcripts in these cells, as measured by quantitative PCR. Tissue analyses revealed expression of the contiguous exon 1N-8 transcript in prostate and in brain. A faint exon 7-lacking transcript is expressed in testis and perhaps in breast. No exon 1N transcript was detectable in human liver, confirming our previous quantitative PCR results.

To summarize our RT-PCR results, each of the three SHBG gene promoters regulates the expression of at least four similar alternatively spliced transcripts. The largest contains contiguous exon 1-8 sequences, while the other three lack exons 7, 6 and 7, and exons 4, 6, and 7, respectively. It is probable that minor species lacking exon 4 and exon 6 alone are also generated, and we speculate there are smaller minor transcripts that probably lack other combinations of exons 4, 6, and 7. It is important to note that exon 4 contains steroid binding and dimerization domains (4). Because the R_{SHBG} binding domain is retained within conserved exon 3, our results raise the interesting possibility that exon 4-lacking transcripts encode SHBG isoforms that could compete for R_{SHBG}, thereby regulating SHBG-mediated steroid signaling through R_{SHBG}. Exon 4-lacking transcripts that contain exon 1L still would retain the signal peptide and therefore could be secreted as a competitive SHBG isoform for R_{SHBG}. Our future studies will be designed to explore the biologic importance of these multiple SHBG transcripts.

Regulation of gene expression in LNCaP cells by SHBG.

Our second major focus during this past year has been to determine whether SHBG expression can affect overall gene expression in LNCaP cells. In our initial proposal, we outlined a series of reporter assays that would be used to determine the effects of SHBG expression on a few androgen responsive genes and constructs that were sensitive to PKA activation. The availability of microarray technology at rapidly falling costs, made microarray analysis a preferable approach for determining the effects of SHBG on gene transcription. As we reported previously, **we generated an LNCaP cell line, L5S2, that expresses and secretes abundant amounts of SHBG when treated with the inducing agent, PonA** (Figures 4a and 4b, from prior years' progress report). We also generated a sister cell line, L5V4, a vector control that lacks the SHBG insert.

L5S2 and L5V4 cells were seeded in medium containing 10% charcoal stripped fetal calf serum. Plated cells were divided into two groups, one treated with the inducing agent, PonA (10uM), and the other treated with the carrier ethanol, for 24 hr. Triplicate wells from each group of cells were then treated for an additional four, eight, 12, 24, and 48 hours with DHT.

This strategy gave us the following treatment conditions (each condition was performed in triplicate)-

1. L5S2 negative treatment control (carrier treated only)*
2. L5S2 PonA treated alone*
3. L5S2 PonA treated, DHT treated 4 hours*
4. L5S2 PonA treated, DHT treated 8 hours
5. L5S2 PonA treated, DHT treated 12 hours
6. L5S2 PonA treated, DHT treated 24 hours*
7. L5S2 PonA treated, DHT treated 48 hours

8. L5V4 negative treatment control (carrier treated only)*

9. L5V4 PonA treated alone*
10. L5V4 PonA treated, DHT treated 4 hours*
11. L5V4 PonA treated, DHT treated 8 hours
12. L5V4 PonA treated, DHT treated 12 hours
13. L5V4 PonA treated, DHT treated 24 hours*
14. L5V4 PonA treated, DHT treated 48 hours

Total cellular RNA was prepared from each of the above triplicate samples.

Due to the expense of microarray analysis, we chose to analyze only one sample from each of the treatment conditions denoted above with a "*". In addition, we analyzed single samples from HepG2 liver cancer cells and a constitutive SHBG overexpressing clone, HepG2myc23, as well as from MCF-7 breast cancer cells, a constitutive SHBG overexpressing clone, MCF-7myc23, and the same two cell lines treated with 10nM estradiol for 4 or 24 hours. Each total cellular RNA sample was pretreated with RNase-free DNase. Total cellular RNAs were sent to our colleagues at the Yerkes Genomics Core Facility at Emory University. RNAs were analyzed by Agilent Bioanalyzer to check the RNA qualities, all of which passed stringent controls. Samples were labeled and hybridized to Affymetrix Human Genome U133 Plus 2.0 Array chips. Raw data was collected by GCOS software and analysis of the microarray data was performed using GeneSpring software. The quality of data, as measured by internal controls and housekeeping genes, was excellent.

A general view of the scope of this work is presented in Figure 4C, which shows the relationship between the 16 samples based on their gene profiles. As expected, there are clusters between the LNCaP, HepG2, and MCF-7 cells. Furthermore, L5S2 samples cluster together, as do L5V4 samples. Similarly, the constitutive SHBG expressing MCF-7myc23 samples cluster together, as do the parental MCF-7 samples.

We are currently performing a detailed analysis of the expression data to determine which genes are specifically regulated by SHBG alone, and by activation of the steroid-SHBG-R_{SHBG} signaling pathway. In Figures 4D-4F, we present a preliminary analysis of our raw data, showing those genes whose expression displays the greatest induction, or repression in L5S2 cells compared to L5V4 vector control cells treated with PonA alone for 24 hours, PonA and then DHT for 4 hours, and Pon A and then DHT for 24 hours.

L5S2 cells induced with 10uM PonA for 24 hours had a 212-fold increase in SHBG expression compared to similarly treated L5V4 cells. They also exhibited a 50- to 100-fold overexpression of 10 genes, one of which is a myosin light chain kinase, while the nine others have not been functionally identified. Many of the most repressed genes appear to be involved in various metabolic processes, eg. a glutamatekainate receptor subunit, so the authenticity of these genes' reduced expression needs to be further established.

L5S4 cells induced with 10uM PonA for 24 hours and then treated with 10nM DHT for an additional 4 hours had a 608-fold increase in SHBG expression compared to similarly

treated L5V4 cells. They also exhibited a 50- to 281-fold overexpression of 15 genes, including contactin 1, a growth differentiation factor, and 13 genes of unknown function. The most repressed genes also appear to be involved mostly in metabolic processes.

L5S4 cells induced with 10uM PonA for 24 hours and then treated with 10 nM DHT for an additional 24 hours had a 394-fold increase in SHBG expression compared to similarly treated L5V4 cells. They also exhibited a 50- to 266-fold overexpression of 6 genes, including an endothelian receptor, a melanoma associated gene, and four genes of unknown function. As above, the most repressed genes appear to be involved mostly in metabolic processes.

A more detailed analysis of our raw data should reveal whether members of the PKA pathway, specifically, and other signaling pathways in general are affected by conditions that activate R_{SHBG}. Furthermore, we are in the process of comparing those genes that are induced by SHBG alone in LNCaP cells are also affected in HepG2 or MCF-7 cells. These findings will be confirmed by quantitative PCR experiments.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstration that human SHBG gene expression differs between liver, testis, prostate and breast tissue.
- Demonstration that human SHBG gene expression differs between HepG2 liver, LNCaP prostate, and MCF-7 breast cancer cell lines.
- Demonstration that SHBG expression in cancer cell lines can differ from corresponding normal tissue.
- Quantitation of SHBG gene expression arising from each of three human SHBG gene promoters in LNCaP, HepG2, MCF-7, 184, MB231 cell lines and in normal prostate, testis, liver and breast tissue.
- Sequence analysis revealed that transcripts derived from the novel upstream promoter encode a short, nine amino acid long peptide, unless these transcripts undergo a novel form of translation.
- Each of the three human SHBG gene promoters gives rise to four main SHBG transcripts, the largest containing contiguous exon 1-8 sequences, and alternative splicing resulting in transcripts that lack exons 7, exons 6 and 7, and exons 4, 6, and 7. Expression of these transcripts appears to be tissue dependent, and cell line dependent.
- The discovery of 1L-containing transcripts that lack exon 4, which contains steroid binding and dimerization domains, is significant. This transcript retains the secretion signal peptide in exon 1L, and the receptor-binding domain within exon 3. If translated, this isoform could be involved in the local regulation of steroid signaling through R_{SHBG}.
- Discovery of minor SHBG gene transcripts that await detailed characterization. Size analysis of RT-PCR bands suggests the existence of transcripts that lack exon 4 alone and exon 6 alone.

- SHBG alone is a potent stimulator/repressor of a number of genes. This new and exciting finding opens up new ways in which to think about how signaling through this pathway occurs.
- SHBG affects DHT induction of genes in LNCaP cells after both 4 hours and 24 hours of DHT treatment. These result are consistent with our main hypotheses posed at the beginning of these studies, namely that SHBG modulates steroid signaling through R_{SHBG} and that SHBG can act as an intracellular buffer for steroids. We are analyzing our microarray data in detail to determine which genes may be modulated by R_{SHBG}, and which androgen responsive genes may be affected by the presence of SHBG in LNCaP cells.

REPORTABLE OUTCOMES:

1. Kahn SM, Hryb DJ, Nakhla AM, Romas NA, Rosner W. Immunohistochemical and in situ detection of sex hormone-binding globulin (SHBG) expression in breast and prostate cancer: Implications for hormone regulation. "Hormonal Carcinogenesis, vol. 4." (2005) Jonathan Li, Sara Li, Antonio Llombart-Bosch, editors. Springer, publisher. pp.508-514

2. Poster Presentation- Era of Hope Conference. Philadelphia, Pa. June 8-11, 2005
HUMAN SEX HORMONE-BINDING GLOBULIN (SHBG) GENE EXPRESSION: UTILIZATION OF MULTIPLE PROMOTERS AND COMPLEX ALTERNATIVE SPLICING OF TRANSCRIPTS
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3. Poster Presentation- Society for Basic Urologic Research Conference, Miami, Fla. December 1-4, 2005
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CONCLUSIONS:

We have shown that the SHBG expression pattern in human tissues and cancer cell lines is complex. The SHBG gene utilizes at least three different promoters. Each promoter generates four or more transcripts, a contiguous full length transcript containing eight exons, and alternatively spliced transcripts that lack exon 7, exons 6 and 7, and exons 4, 6, and 7. We also detected minor transcripts of low abundance by RT-PCR analysis, and these await sequence analysis. Our discovery of a novel transcript that is expressed from

the downstream liver promoter, and which lacks exons 4, 6, and 7, is exciting. This transcript retains the signal peptide within the first exon, and the R_{SHBG} binding domain within exon 3, but lacks the steroid binding and dimerization domains within exon 4. If translated, this SHBG isoform could be involved in the regulation of membrane based steroid signaling by competitively binding to R_{SHBG}.

Using quantitative PCR analysis of SHBG expression in tissues and cell lines we have confirmed that the liver has abundant expression of the secreted form of SHBG, and the testis has abundant expression of the exon 7-lacking transcript originating from the middle promoter. As anticipated, SHBG promoter utilization displays some tissue specificity. Focusing on the prostate, our study found that overall SHBG expression in LNCaP cells is approximately 7-fold higher than expression in normal prostate. The majority of SHBG transcripts in LNCaP cells are derived from the middle (testis) promoter, with 70-80% lower expression from the downstream liver promoter and the novel, upstream promoter. Normal prostate tissue, on the other hand, shows SHBG expression predominantly from the downstream liver promoter with 42% and 81% lower expression from the middle, and upstream novel promoters, respectively. This altered promoter utilization was also seen in the liver and breast cancer cell lines, as compared to normal tissue.

Of all the samples tested, the highest expression of transcripts from the novel, upstream promoter was seen in LNCaP cells. Because all transcripts derived from the novel, upstream promoter have a very short open reading frame of nine amino acids, the functional significance of this transcript in LNCaP cells remains to be determined. It is interesting to note that this novel promoter is virtually silent in normal liver tissue, whereas it is utilized, albeit at very low levels, in HepG2 liver cancer cells.

The microarray analysis of the effects of SHBG on gene expression in LNCaP cells is quite provocative. It is evident that SHBG alone can have profound effects on gene expression. This observation invokes a steroid-independent function for locally expressed SHBG in the prostate. A detailed analysis of our microarray results are in progress and will give us a better clue as to which signaling pathways are influenced by SHBG itself.

We also addressed our main hypotheses that locally expressed SHBG can influence the DHT response of LNCaP cells by comparing the expression profiles of SHBG overexpressing cells treated with DHT for 4 and 24 hours to those of similarly treated nonoverexpressing cells. Our results show that SHBG overexpression affects the DHT response of LNCaP cells. Genes such as myosin light chain kinase, an endothelian receptor, and a melanoma associated gene are among those genes that show greatly elevated expression in SHBG overexpressers. We are currently performing a detailed analysis of these microarray results to determine whether induction or repression of DHT responsive genes is modulated by SHBG, and to identify specific genes that are induced or repressed following activation of the SHBG-R_{SHBG} pathway. We anticipate that these results will provide support for a model in which locally expressed SHBG can act both as a buffer for DHT, and as an autocrine/paracrine activator of R_{SHBG}.

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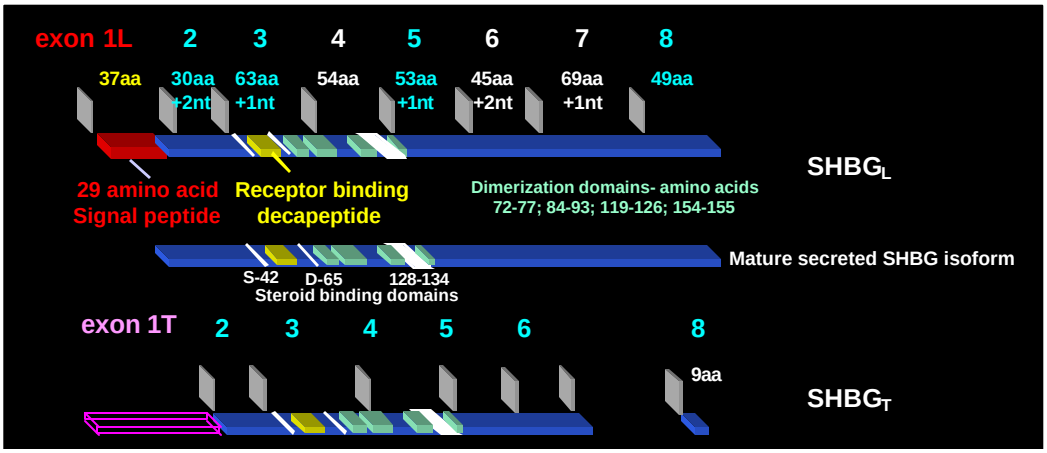
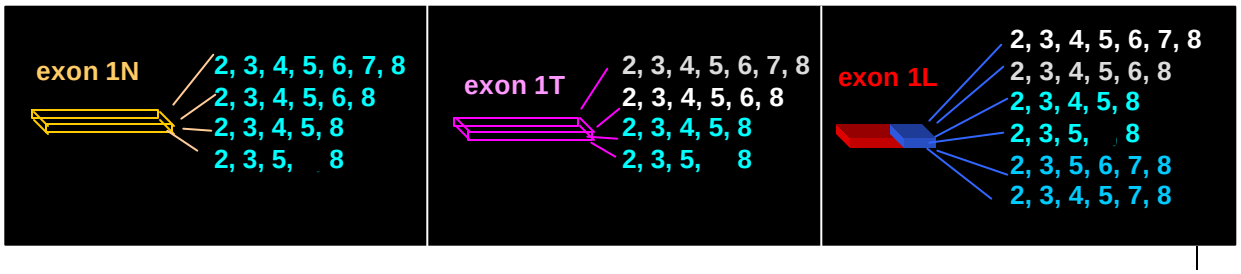
Figure 1. Discovery of novel SHBG gene mRNA transcripts.



A. Exon structure and functional domains of the two previously described major human SHBG transcripts, SHBG$_L$ and SHBG$_T$. SHBG$_L$ is the major mRNA synthesized in the liver, and encodes the secreted form of SHBG found in the plasma. SHBG$_L$ is eight exons long (exon number and length in amino acids is given above) and contains a 29 amino acid leader sequence (red) which is encoded within exon 1L. The leader sequence is cleaved prior to the secretion of the mature form of SHBG. Mature SHBG exists in the plasma as a homodimer, which is mediated by sequences within exons 3 and 4 (green). Steroid binding occurs in a pocket bounded by amino acids within exons 3 and 4 (white). The R_{SHBG} binding site is within the decapeptide sequence found in exon 3 (yellow). SHBG$_T$ is the major SHBG transcript found in the testis. It is transcribed from a promoter that lies approximately 2kb upstream of the SHBG$_L$ promoter. SHBG$_T$ contains different exon 1 sequences (purple) and lacks a signal peptide. Because SHBG$_T$ lacks exon 7 sequences, a frameshift occurs within exon 8, thereby creating an alternative reading frame at the carboxyl terminus consisting of nine amino acids.



B. Schematic representation of 14 currently known human SHBG mRNAs. Shown are the exon structures of SHBG transcripts derived from the novel upstream promoter (left), the SHBG$_T$ promoter (middle), and the SHBG$_L$ promoter (right). In total, we have detected 14 SHBG gene transcripts, four of which have been described in the literature, and ten of which are novel. Four novel SHBG transcripts contain exon 1N (left, light blue), a full length transcript containing contiguous exon 2-8 sequences, and three additional alternatively spliced transcripts lacking exons 7, 6 and 7, and 4, 6, and 7, respectively. Four SHBG transcripts contain exon 1T (middle), including the major exon 7-lacking transcript (white), a previously described full length minor transcript (gray) and two novel alternatively spliced transcripts that lack exons 6 and 7, and 4, 6, and 7, respectively (light blue). Six SHBG transcripts contain exon 1L, including the major full length transcript encoding SHBG$_L$ (white), a previously described minor transcript lacking exon 7(gray), two novel transcripts which have been confirmed by DNA sequence analysis to lack exons 6 and 7, and 4, 6, and 7, respectively (light blue), and two additional minor transcripts that we have detected by RT-PCR analysis and are currently presumed to lack exons 4 and 6, respectively (blue).

Figure 2. Quantitation of Human SHBG Expression in Cell Lines and Tissues

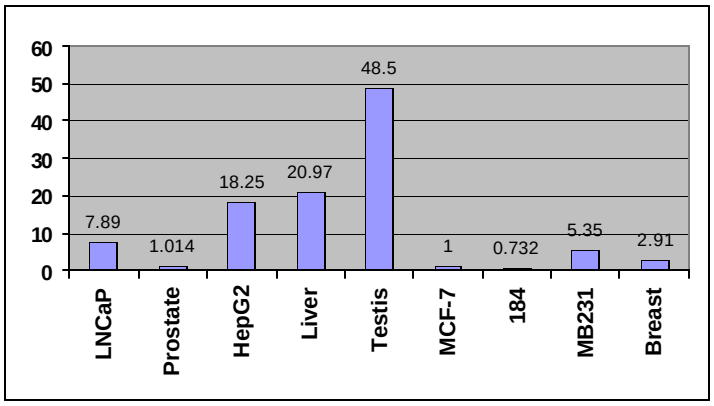


Figure 2A- Relative overall SHBG expression levels in human cell lines and tissues. Quantitative RT-PCR was performed using primers specific for conserved SHBG transcript regions within exons 2 and 3. Average expression levels are given above the data bars, and are normalized to MCF-7 expression levels. Experiments were performed in triplicate, on either two or three RNA samples.

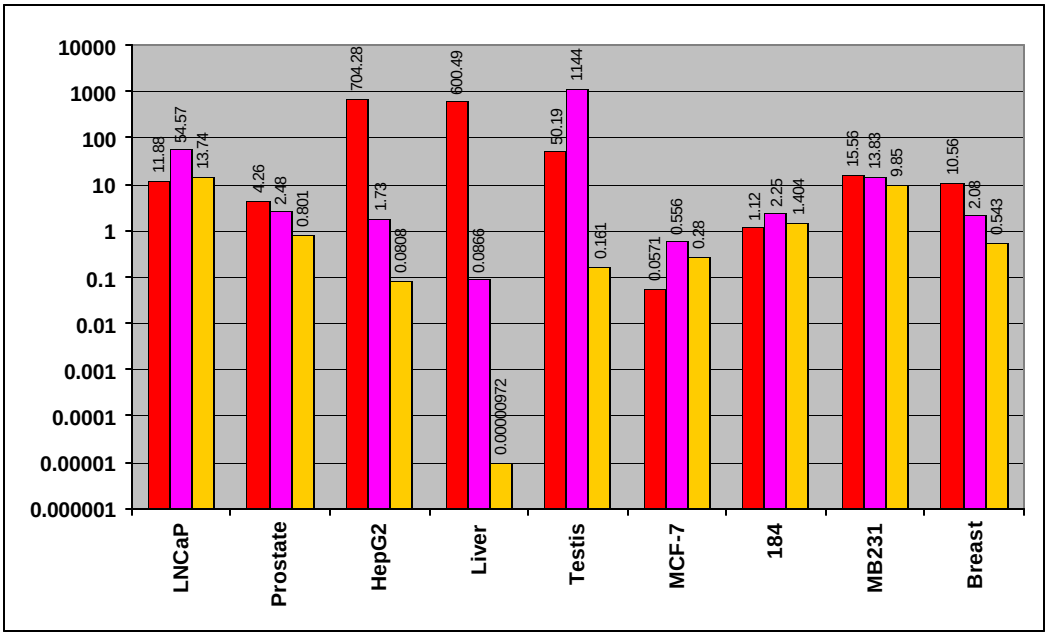


Figure 2B SHBG gene promoter utilization. Quantitative RT-PCR was performed on human cell lines and tissues to determine relative expression of SHBG gene transcripts originating from the downstream promoter (PL, red), the intermediate promoter (PT, pink), and the novel upstream promoter (PN, orange). Primer sets were specific for SHBG exons 1L and 2, 1T and 2, and 1N and 2, respectively. Experiments were performed in triplicate, on either two or three RNA samples. Expression levels are normalized to overall MCF-7 expression levels from Figure 2A. Note- this is a logarithmic scale. Expression levels are denoted above each data bar.

Figure 3. RT-PCR analysis of SHBG transcripts in human cell lines and tissues.

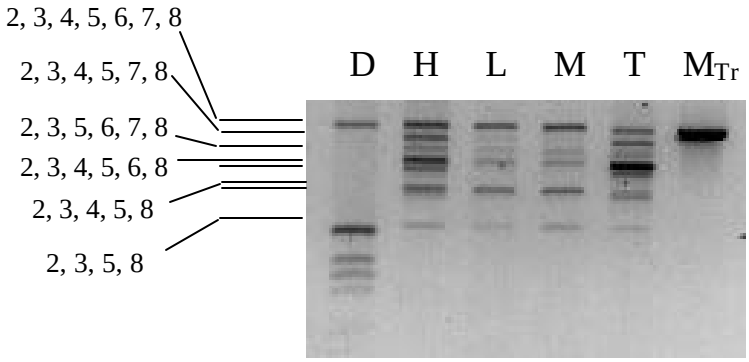


Figure 3A. Splicing of human SHBG transcripts involves complex alternative splicing of exons 4, 6, and 7. First strand oligo-dT primed cDNAs from MCF-7 breast (M), LNCaP prostate (L), HepG2 liver (H), normal testis tissue(T), and an MCF-7 clone overexpressing $SHBG_L$ (M_{Tr}) were amplified using a forward primer specific for exon 2 and the same exon 8 reverse primer as above. On the left are the exon structures of the RT-PCR bands, as determined by DNA sequence analysis D: DNA size marker.

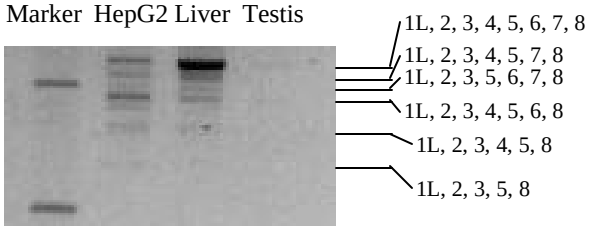


Figure 3B. Exon 1L- specific RT-PCR amplification reveals alternative splicing of exons 4, 6, and 7 in HepG2 cells and in liver tissue. RT-PCR was performed using a forward primer specific for $SHBG_L$ exon 1 and a reverse exon 8 primer. HepG2 cells and normal liver tissue gave similar sized bands with different intensities, while testis tissue gave faint bands. MCF-7 and LNCaP did not give any bands (data not shown), however these experiments are currently being repeated. Exon structures of six of the eight bands are given on the right. Four have been confirmed by sequence analysis. Sequencing of the minor bands awaits further analysis, although the structures of two transcripts (the exon 6-lacking and exon 4-lacking transcripts) are predicted based on the band sizes. D: DNA marker.

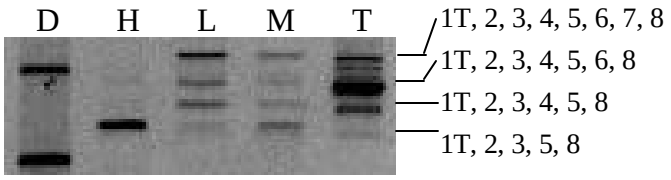


Figure 3C. RT-PCR analysis of Exon 1T- containing transcripts. RT-PCR was performed using a forward primer specific for $SHBG_T$ exon 1 and a reverse exon 8 primer. LNCaP, MCF-7, and testis tissue gave similar sized bands with different intensities, while HepG2 appeared to mostly express a transcript that lacks exons 4, 6, and 7. Exon structures of the four major bands are given on the right. Sequencing of the minor bands awaits further analysis. D: DNA marker.

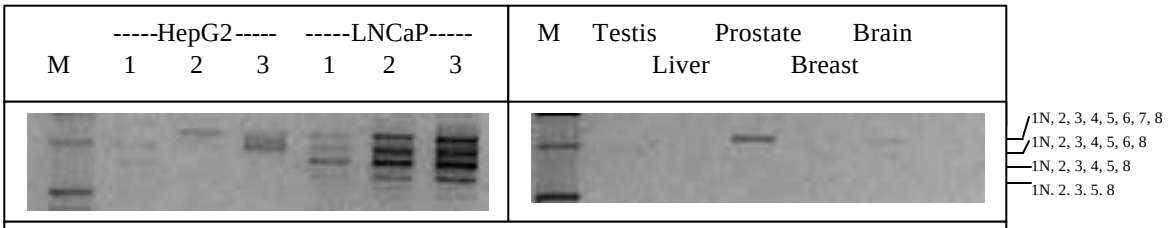
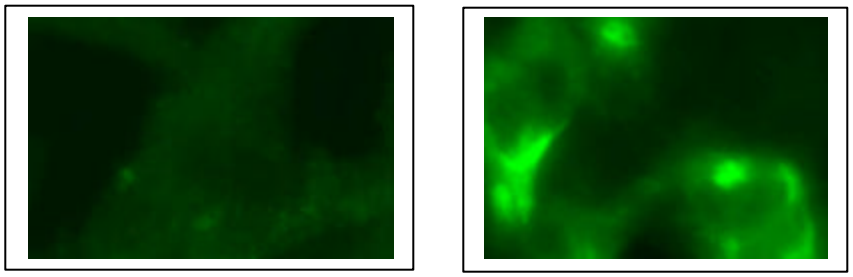


Figure 3D. RT-PCR analysis of Exon 1N- containing transcripts. RT-PCR was performed using a forward primer specific for $SHBG_N$ exon 1 and a reverse exon 8 primer. Three independent plates of HepG2 and LNCaP cells were processed into cDNAs and analyzed by RT-PCR. cDNAs from normal testis, liver, prostate, breast and brain tissue were similarly analyzed. LNCaP cells exhibited four major RT-PCR bands corresponding to contiguous exon 1N-8 sequences, and transcripts lacking exons 7, 6 and 7, and 4, 6, and 7, respectively in all independent isolates. Exon structures were confirmed by sequence analysis. The HepG2 isolates gave different patterns. Sequence analysis has not yet been performed on these bands. Prostate tissue gave rise to a single major band, corresponding to contiguous exon 1N-8 sequences, while brain tissue gave a fainter band. Testis gave a band that comigrated with the exon 7-lacking RT-PCR transcript. Exon 1N containing RT-PCR bands were not detected by RT-PCR in liver and breast tissue. D: DNA marker.

Figure 4. Effects of SHBG Induction on Overall Gene Expression in LNCaP Cells.



A: L5S2 uninduced L5S2 induced

Figure 4A. Immunohistochemical detection of SHBG in inducible L5S2 cells, and effects of DHT and E2 treatment. L5S2 cells were plated on glass slides. Cells were treated with carrier alone (left) or induced to express SHBG by treatment with 10uM Pon A for 24 hours (right). Cells were fixed, exposed to an anti-human SHBG polyclonal antibody and then developed with a rabbit anti-mouse IgG1 linked to the green fluor, Alexa-488. Relative SHBG staining is shown (control cells exhibited very low level staining, and this staining was not affected by PonA treatment (data not shown)).

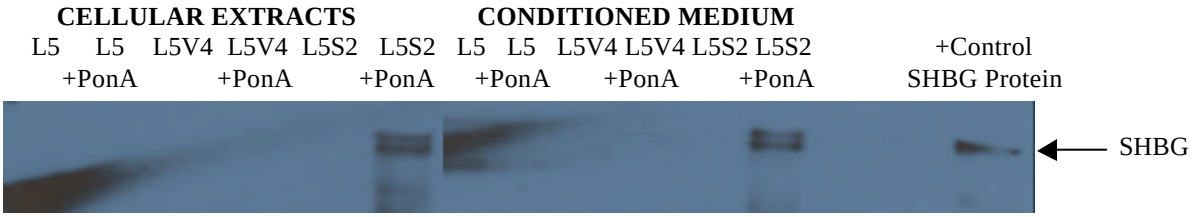
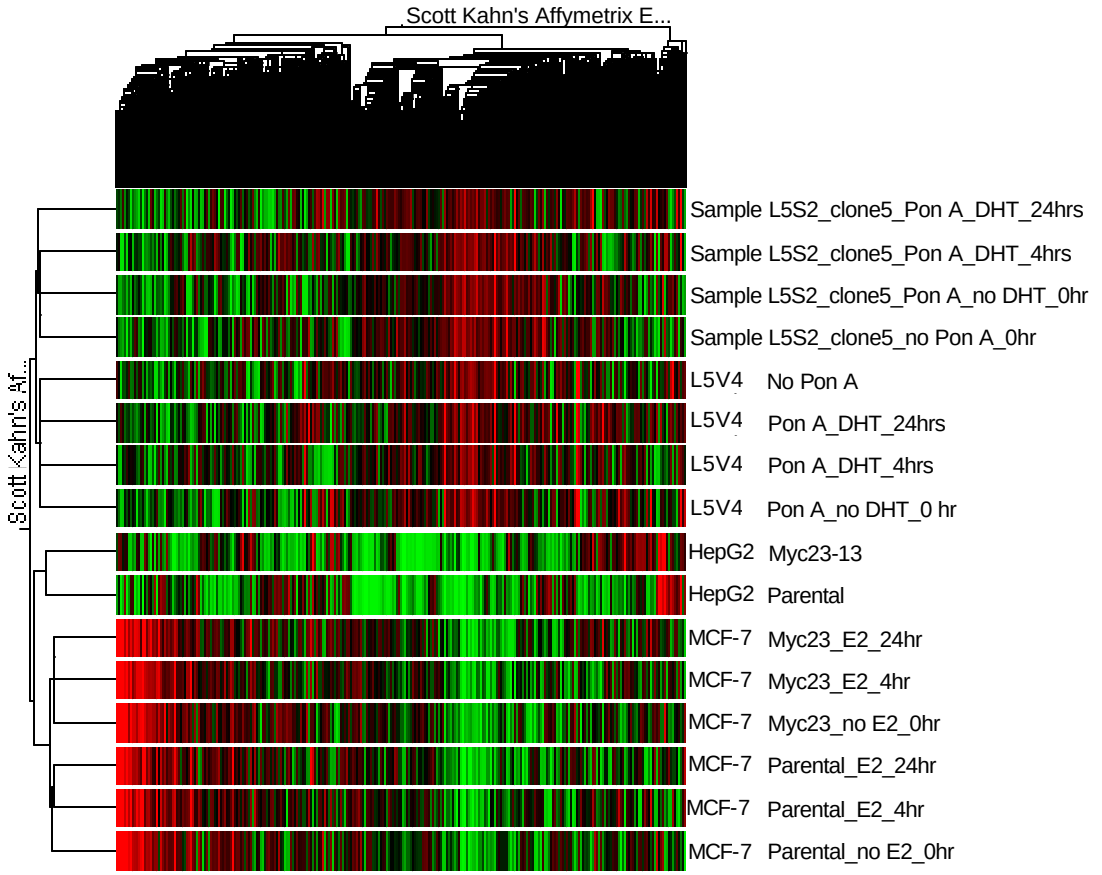
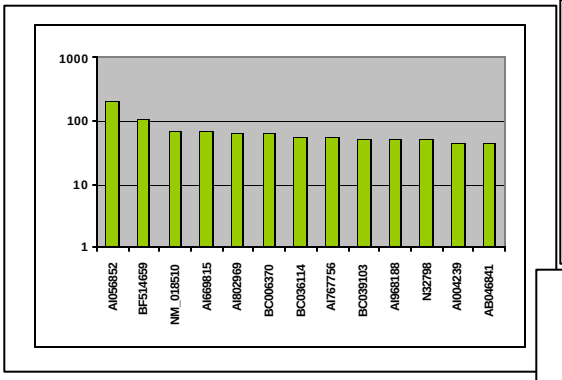


Figure 4B: PonA induces SHBG protein expression in L5S2 cells. Parental L5, vector control L5V4, and inducible L5S2 cells were plated in duplicate in 6 well dishes at 75% confluence. After incubating for 48 hours, cells in one well were exposed to 10uM PonA for 24 hours, while control, unexposed cells were mock treated with solvent. Total cellular protein was prepared, and conditioned medium was isolated and spun down to remove cells and cellular debris. Cellular extracts and 30ul aliquots of conditioned medium were analyzed by Western blot. 10ng of purified SHBG was loaded in the positive control lane. This short exposure shows inducible expression of SHBG in L5S2 cells, and secretion. Two SHBG bands are visible in the induced L5S2 lanes, similar findings were obtained using a constitutively expressed SHBG full length construct in LNCaP cells (data not shown). The reason for this observation is unclear at the present time.



Colored by: Scott Kahn's Affymetrix Experiment (Default Interpretation)
Gene List: all genes (54675)

Figure 4C. Similarity Tree of Cell Lines and Treatment Conditions Based on Gene Expression Profiles. Sixteen different LNCaP, HepG2, and MCF-7 samples were analyzed by microarray analysis. Effects of SHBG induction in LNCaP cells were investigated using the inducible LNCaP cell line, L5S2. Total cellular RNA was prepared from L5S2 cells, and its sister vector control cell line, L5V4, as well as from cells that had been treated with the inducing agent, PonA (10uM) for 24 hours, PonA and then 10nM DHT for 4 hours, and PonA and then 10nM DHT for 24 hours. Total cellular RNA was also prepared from HepG2 liver cancer cells, and a constitutive SHBG overexpressing clone, HepG2myc23. In addition, Total cellular RNA was prepared from MCF-7 breast cancer cells and a constitutive SHBG overexpressing clone, MCF-7myc23, as well as from the same two cell lines treated with 10nM estradiol for 4 or 24 hours. Total cellular RNA was pretreated with RNase-free DNase. Following this, the samples were cellular RNAs were sent to our colleagues at the Yerkes Genomics Core Facility at Emory University. RNAs were analyzed by Agilent Bioanalyzer to check the RNA quality. Samples were labeled and hybridized to Affymetrix Human Genome U133 Plus 2.0 Array chips. Raw data was collected by GCOS software and analysis of the microarray data was performed using GeneSpring software. Shown is an expression tree which compares the 16 different samples analyzed in this study, with respect to overall gene expression profiles.



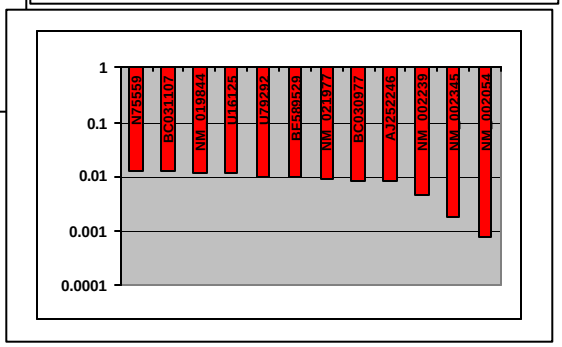
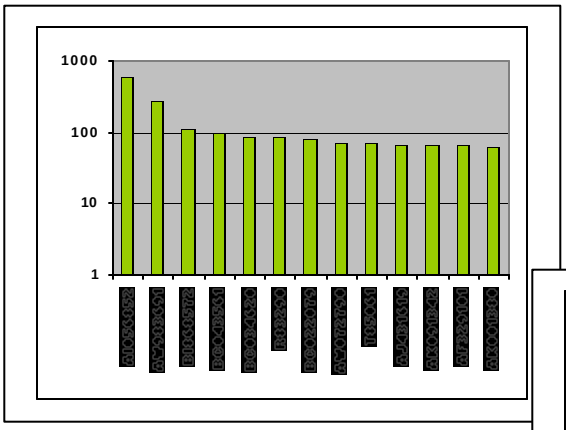


Figure 4D- Genes whose expression in L5S2 cells treated with PonA for 24 hours is increased by 44-fold or greater (green, right) or is decreased by 80-fold or greater (red, below) as compared to PonA treated vector control L5V4 cells.



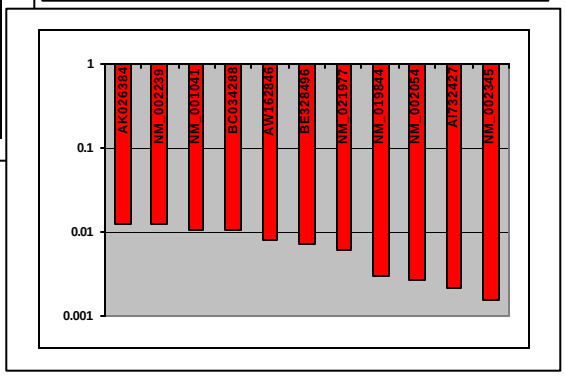


Figure 4E -Genes whose expression in L5S2 cells treated with PonA and 10nM DHT for 4 hrs is increased by 60-fold or greater (green, right) or is decreased by 80-fold or greater (red, below) compared to similarly treated vector control L5V4 cells.

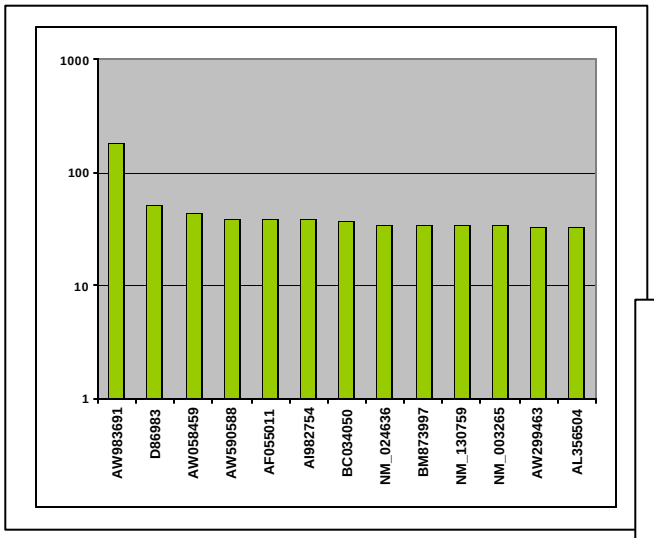


Figure 4F -Genes whose expression in L5S2 cells treated with PonA and 10nM DHT for 24 hrs is increased by 33-fold or greater (green, right) or is decreased by 80-fold or greater (red, below) compared to similarly treated vector control L5V4 cells.